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Research Note—

Phylogenetic Analysis of the Sigma 2 Protein Gene of Turkey Reoviruses

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SUMMARY. The open reading frame of the S3 segment encoding the σ2 protein of four turkey reovirus field isolates was analyzed for sequence heterogeneity. The turkey reoviruses we present here have a 97% amino acid identity to turkey NC 98. The S3 nucleotide and amino acid sequence similarity was ≤61% and 78%–80%, respectively, when compared to the chicken reovirus isolates. Comparison of amino acid sequences from chickens and turkeys with that of a duck isolate revealed a 53% and 55% similarity, respectively. Phylogenetic analyses, based on both nucleotide and amino acid sequence, resulted in three major groups among the avian reoviruses; these groups were clearly separated by species. The results of this study provide further evidence, based on the deduced σ2 sequence, that turkey reoviruses form a distinct, separate group relative to chicken and duck isolates. In addition, as a result of the limited sequence identity with their avian counterparts, turkey reoviruses could potentially be considered a separate virus species within subgroup 2 of the *Orthoreovirus* genus.

RESUMEN. *Nota de Investigación*—Análisis filogenético del gen que codifica para la proteína Sigma 2 de los reovirus de pavo.

Con el fin de determinar la heterogeneidad entre secuencias, se analizaron los marcos de lectura continuos de los segmentos S3 que codifican para la proteína sigma 2 en cuatro aislamientos de campo de reovirus en pavos. Los reovirus de pavos de este estudio presentan una identidad del 97% en la secuencia de aminoácidos con la cepa NC 98 de pavos. Los porcentajes de similitud de las secuencias de nucleótidos y aminoácidos fueron de ≤61% y de 78% a 80%, respectivamente, cuando se compararon con las secuencias de aislamientos de reovirus de pollo. Las secuencias de aminoácidos de aislamientos de pollos y pavos revelaron porcentajes de similitud de 53% y 55%, respectivamente, cuando se compararon con un aislamiento de pato. Los análisis filogenéticos basados en las secuencias de nucleótidos y aminoácidos resultaron en tres grupos principales de reovirus aviares que están separados claramente de acuerdo con la especie. Los resultados de este estudio proporcionan evidencia de que los reovirus de pavo forman un grupo distinto y separado de los aislamientos de pollo y pato con base en las secuencias deducidas de aminoácidos. Además, debido a que la identidad de secuencias limitada con sus contrapartes aviares, los reovirus de pavo pueden considerarse como una especie separada dentro del subgrupo 2 del género *Orthoreovirus*.

Key words: turkey reovirus, sigma 2 protein, S3 gene

Abbreviations: ARV = avian reovirus; ATCC = American Type Culture Collection; ICTV = International Committee on Taxonomy of Viruses; NBV = Nelson Bay virus; PEMS = poult enteritis mortality syndrome; RT-PCR = reverse transcription–polymerase chain reaction

Avian reoviruses, along with mammalian reoviruses, comprise the genus *Orthoreovirus* in the family *Reoviridae*. These viruses contain 10 dsRNA genome segments enclosed within a nonenveloped, icosahedral double capsid of approximately 80 nm (5,15). The genome segments can be separated based on electrophoretic mobility into three large (L1, 2, 3), three medium (M1, 2, 3), and four small

(S1, 2, 3, 4) segments that code for proteins λ1, λ2, λ3, μ1, μ2, μNS, σ3, σ1, σ2, and σNS, respectively (17,20,22). The S3 segment of avian reovirus encodes the σ2 protein and is analogous to the mammalian reovirus S4 segment encoding the σ3 protein. The σ2 protein is an outer capsid protein that carries group-specific neutralizing epitopes (21). It also binds double-stranded RNA and has been identified as a zinc metalloprotein (10). Avian reoviruses promote syncytial formation in cultured cells (9) but do not hemagglutinate like their mammalian counterparts (2,11). Three subgroups of orthoreoviruses have been identified by the International Committee on Taxonomy of Viruses (ICTV). Avian reoviruses and a mammalian isolate, Nelson Bay virus (NBV), are classified in the fusogenic subgroup 2 (8). Criteria for orthoreovirus species demarcation include nucleotide or amino acid identity of greater than 75% and 85%, respectively, within a species, or less than 60% and 65%, respectively, between a species (8).

Avian reoviruses (ARVs) are a diverse group of poultry pathogens, the virulence of which varies greatly among isolates within different hosts. ARVs have been isolated from turkeys with poult enteritis and mortality syndrome (PEMS)(4) as well as from chickens and ducks. These isolates have been associated with enteric and respiratory disease (1), viral arthritis/tenosynovitis (2), malabsorption, and stunting syndrome (12). Not all reoviruses are highly virulent, as they can be isolated from chickens or turkeys exhibiting no clinical signs of disease. Recently, we isolated and sequenced the first turkey reovirus S3 gene for isolate NC 98 (GenBank accession no. AF465799) (6). Phylogenetic analysis of the S3 gene and σ2 protein sequences of NC 98 places it in a distinct and separate group from other avian and NBV isolates within the subgroup 2 clade (6). In this study we present phylogenetic analysis of the S3 gene from four turkey field isolates.

MATERIALS AND METHODS

Viruses. Reovirus field isolates PEMS 85, TX 98, and TX 99 were isolated at the Poultry Diagnostic and Research Center at the University of Georgia, from the intestines/feces of turkey poults exhibiting enteritis. Viral strain ATCC TEV was purchased from the American Type Culture Collection (ATCC #VR-818); Manassas, VA). All viruses were propagated in primary chicken embryo liver cells for a total of four passages. Upon the development of 70%–80% cytopathic effect, observed as syncytial formation, the cell cultures were frozen and stored at −80 C. Cell cultures were frozen and thawed three times prior to subsequent cell culture passage.

RNA extraction. Total viral RNA was extracted from primary chicken embryo liver cell passages using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations.

Reverse transcription–polymerase chain reaction. A cDNA corresponding to the S3 gene was produced by reverse transcription–polymerase chain reaction (RT-PCR) using SuperScript™ II RNase H-RT and Platinum®Taq DNA polymerase (Invitrogen, Carlsbad, CA) with previously published S3F and S3R primers (6). The 1.1-kb amplified products were separated on a 1.0% agarose gel, stained with ethidium bromide, and visualized with an ultraviolet transilluminator. The fragment was excised, purified with the QIAEX II gel extraction kit (Qiagen, Inc., Valencia, CA), eluted in diethylpyrocarbonate-treated water, and stored at −80 C until sequenced.

Direct nucleotide sequencing of amplified products. Gel-purified PCR products were sequenced using double-stranded DNA sequencing with fluorescently labeled dideoxynucleotides and Taq polymerase and performed on an ABI 3100 automated sequencer (Applied Biosystems, Inc., Foster City, CA) (16). PCR primers S3F and S3R were used to sequence as well as conserved internal S3 gene primers as needed to complete sequencing. Primer sequences used for sequencing are available upon request.

Sequence analysis. Nucleotide, predicted amino acid sequence analysis, and multiple alignments of the S3 gene and sigma 2 protein were performed using CLUSTAL V (Lasergene, v. 5.0, DNASTAR, Madison, WI). Sequences for the S3 genes of previously published avian reoviruses were obtained from GenBank and had the following accession numbers: NC98-AF465799, chicken S1133-AO020642, chicken 1733-AF004856, chicken 138-AF059721, and muscovy duck 89026-AJ006476. Sequences obtained for turkey isolates described here have been submitted to GenBank and assigned the following numbers: TX 99-AY444910, TX 98-AY444911, ATCC TEV-AY444912, and PEMS 85-AY444913. Aligned sequences were compared and a phylogram was generated using maximum parsimony analysis with Neighbor-Joining clustering (3,13) and 1000 bootstrap replicates (confidence levels listed in parentheses) in a heuristic search using the Phylogenetic Analysis Using Parsimony (v. 4.10b) software (PAUP) (18).

RESULTS AND DISCUSSION

The nucleotide and deduced amino acid sequences of the S3 segment of TX 99, TX 98, PEMS 85, and ATCC TEV reovirus isolates were compared with previously published avian and mammalian reoviruses belonging to subgroup 2 orthoreoviruses

Table 1. Comparison of nucleotide and deduced amino acid sequences of the S3 gene of subgroup 2 orthoreoviruses.

		% Amino acid identity									
		1	2	3	4	5	6	7	8	9	10
% Nucleotide identity	1. 1133	**100**	99.2	99.2	79.3	60.8	77.9	77.9	77.9	79.3	11.8
	2. 1733	99.5	**100**	100	79.8	61.0	78.5	78.5	78.5	79.8	12.1
	3. 176	99.6	99.9	**100**	79.8	61.0	78.5	78.5	78.5	79.8	12.1
	4. ATCC	65.8	66.2	66.1	**100**	61.9	96.7	95.9	95.9	100	10.7
	5. Duck	53.6	53.9	53.8	56.3	**100**	61.3	61.3	61.3	61.9	10.5
	6. NC 98	64.5	64.9	64.9	96.1	55.2	**100**	96.7	96.7	96.7	10.5
	7. PEMS 85	64.9	65.3	65.2	95.6	55.0	94.7	**100**	100	95.9	10.7
	8. TX 98	64.9	65.3	65.2	95.6	55.0	94.7	100	**100**	95.9	10.7
	9. TX 99	65.8	66.2	66.1	100	56.3	96.1	95.6	95.6	**100**	10.7
	10. NBV	19.4	19.3	19.3	18.2	19.5	19.1	18.4	18.4	18.2	**100**

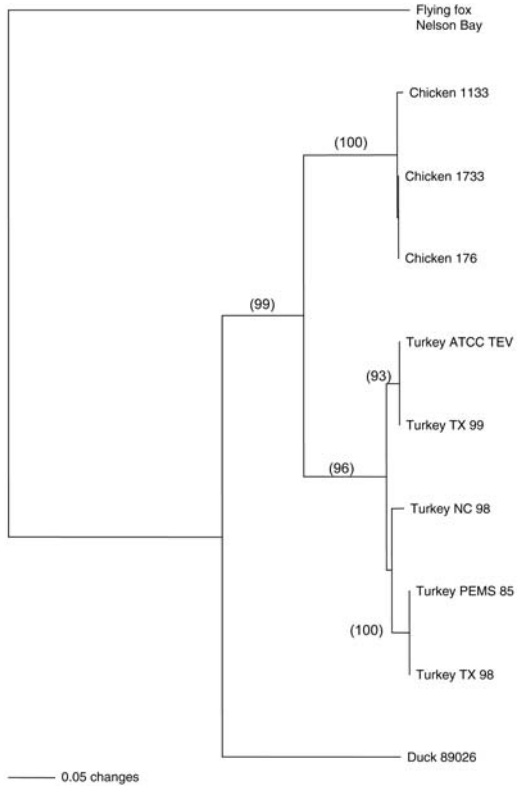

Fig. 1. Phylogenetic relationships among fusogenic subgroup 2 orthoreoviruses based on σ2 protein sequence. Following alignment, a midpoint rooted phylogram was generated using maximum parsimony analysis with Neighbor-Joining clustering and 1000 bootstrap replicates (confidence levels listed in parentheses) in a heuristic search using PAUP 4.0bv10 (18).

(Table 1). The turkey isolates shared a 95% and 97% nucleotide and amino acid similarity, respectively, with the previously reported turkey isolate NC 98. The turkey isolates shared at least a 95% similarity among the isolates, whereas only a 64% identity with the chicken isolates and a 55% identity with the duck isolate at the nucleotide level. The chicken reoviruses shared 83%–99% nucleotide identity among isolates and approximately 53% identity to the duck 89026 isolate. Alignment of the deduced amino acid sequences for the σ2 protein revealed a 95%–99% identity among all turkey isolates and 95%–100% identity among the chicken isolates examined. The turkey isolates shared only a 77%–81% identity with the chicken isolates. Both chicken and turkey isolates shared approximately 60% identity with the duck isolate at the amino acid level. All avian reoviruses shared only a 10%–11% identity with the fusogenic mammalian reovirus, NBV, at the amino acid level.

Phylogenetic analysis of the σ2 protein sequences demonstrated that the avian and the mammalian NBV reoviruses separate from subgroup 1 and 3 orthoreovirus isolates (data not shown) to form subgroup 2 orthoreovirus (Fig. 1). Within the subgroup 2, the turkey isolates form a separate clade from duck, NBV, and chicken isolates. This relationship was also determined using nucleotide sequences of the S3 gene among subgroup 2 orthoreoviruses (data not shown). The same clades were obtained whether distance, maximum likelihood, or parsimony methods were utilized to generate the phylogram (data not shown).

We previously identified a conserved CHCC zinc-binding motif, as well as a putative double-stranded RNA binding motif within the NC 98 σ2

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NC 98       M E V R V P N F H S F V E G I T S S Y I R A P A C W N A K T M W D V E T F H L P D V I K V G N A Y C      50
ATCC TEV    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .      50
PEMS 85     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . N I . . . . . . . . . . . .      50
TX 98       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . N I . . . . . . . . . . . .      50
TX 99       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .      50
Duck        . . . . . . . . . . . . . . . . . A . . . . T . . . . D S R S A . . S D I . Y E . . . . . . . G . . .      50
1133        . . . . . . . . . . . . . . . . . . . L K T . . . . . . Q . A . . T V . . . V . . . . . R . . . . . .      50
1733        . . . . . . . . . . . . . . . . . . . L K T . . . . . . Q . A . . T V . . . V . . . . . R . . . . . .      50
176         . . . . . . . . . . . . . . . . . . . L K T . . . . . . Q . A . . T V . . . V . . . . . R . . . . . .      50

NC98        C S Q C C G V L Y Y G A P P S D G N Y F P H H K C H Q Q Q Y R S D T P L L R Y V R I G S T T E H L L    100
ATCC TEV    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . R . . . . . .    100
PEMS 85     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . R . . . . . .    100
TX 98       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . R . . . . . .    100
TX 99       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . R . . . . . .    100
Duck        . T . . . . . . . . . . . . . T . . I C . . . . . . . . . L S . V . N . . . . F . K . . R . . . . . .    100
1133        . . . . . . . . . . . . T L . A . . . . . . . . . . . . . . . . T . . . . . . . . . . . R . . . . . .    100
1733        . . . . . . . . . . . . T L . A . . . . . . . . . . . . . . . . T . . . . . . . . . . . R . . . . . .    100
176         . . . . . . . . . . . . T L . A . . . . . . . . . . . . . . . . T . . . . . . . . . . . R . . . . . .    100

NC 98       D Q Y A V A L Q T I A D Y Y D E A S H R V A D E A E E D T I A A L D I V T R T E S I R G D Q A V D A    150
ATCC TEV    . . . . . . . . . . . . . . . . . . . . . . . . P . . . . . . . . . . . . . . . . . . . . S . . . . . .    150
PEMS 85     . . . . . . T . . . . . . . . . . . . . . . . . P . . . . . . . . . . . . . . . . . . . . S . . . . . .    150
TX 98       . . . . . . T . . . . . . . . . . . . . . . . . P . . . . . . . . . . . . . . . . . . . . S . . . . . .    150
TX 99       . . . . . . . . . . . . . . . . . . . . . . . . P . . . . . . . . . . . . . . . . . . . . S . . . . . .    150
Duck        . . . . . I . S C . . . H . E Q . A R S R T S M C G G . A L M S . . . I M . . . . L . T . R P . . P    150
1133        . . . . . . . E S . . . H . . . I . Q . M V . . P . N . E V . P . . . . . . . . . . . . S . K T . . P    150
1733        . . . . . . . E S . . . H . . . I . Q . M V . . P . N . E V . P . . . . . . . . . . . . S . K T . . P    150
176         . . . . . . . E S . . . H . . . I . Q . M V . . P . N . E V . P . . . . . . . . . . . . S . K T . . P    150

NC 98       D F W T Y P L E R R S D D S R R D I A A S I W T M I D A S A R S F T L P E C L V S P S L H S R H I F    200
ATCC TEV    . . . . . . . . . . . . . . . . E . . . . . . K . . . . . . . . . . . . . . D . . . . . . . . . V .    200
PEMS 85     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . D . . . . . . . . . . .    200
TX 98       . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . D . . . . . . . . . . .    200
TX 99       . . . . . . . . . . . . . . . . E . . . . . . K . . . . . . . . . . . . . D . . . . . . . . . . V .    200
Duck        . . . . A . . D . . E P . A . S . . . T A G . R . . . . . S Q . Q . V . D . V . . N L . . T . . V .    200
1133        . . . . . R . . . . . . . . . . . . . . . . C . R . . . . . S . . L . . . N . . . . . . . . . S V .    200
1733        . . . . . . . . . . . . . . . . . . . . . . C . R . . . . . S . . L . . . N . . . . . . . . . S V .    200
176         . . . . . . . . . . . . . . . . . . . . . . C . R . . . . . S . . L . . . N . . . . . . . . . S V .    200
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Fig. 2. Predicted amino acid sequence alignment of sigma 2 proteins (S3 gene) from avian reovirus isolates. The putative zinc-finger binding-motif is underlined (amino acids 51–76) and the proposed dsRNA-binding motif is boxed (amino acids 287–293).

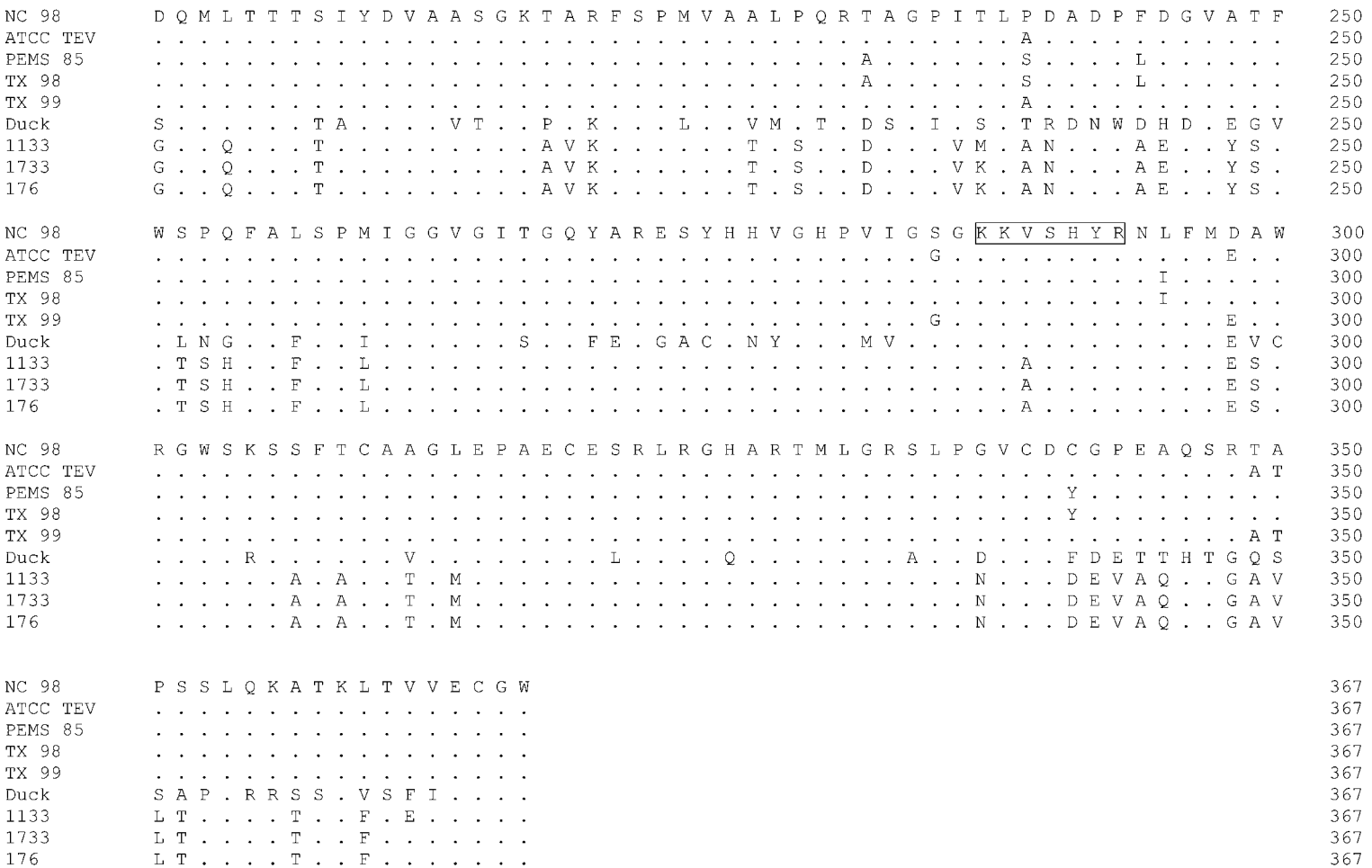

Fig. 2. Continued.

amino acid sequence (6), as was identified in the mammalian σ3 by others (7,14,19,23). Our results indicate that isolates PEMS 85, TX 98, TX 99, and ATCC TEV also contain these motifs (Fig. 2) and that the amino acid sequences are conserved among the turkey isolates examined. Several common amino acid substitutions within the σ2 protein were observed among the turkey and duck reovirus isolates when compared to the chicken isolates examined. At amino acids 62 and 63, chicken isolates contained a threonine and leucine, whereas turkey and duck isolates contained alanine and proline, respectively. The chicken isolates also contained an alanine at amino acid 65, whereas turkey isolates had a serine in this position. These substitutions are located within a putative CHCC zinc-binding domain (14). Within the putative double-stranded RNA binding region, chicken isolates contained an alanine at position 288 and turkey isolates contained a lysine. Other species-specific amino acid substitutions (chkn→tky/duck) were observed at amino acid positions 198 (serine→histidine), 204 (glutamine→leucine), 219 (valine→alanine), 229 (serine→proline), 289 (alanine→valine), 309 (alanine→threonine), and 314 (methionine→leucine). Turkey isolates contained a serine, as did the duck isolate, at position 307, while chicken isolates contained an alanine at 307. Although the substitutions appear to be conserved by species, their significance has not yet been determined.

In this study we report the S3 gene sequences of four turkey reovirus field isolates. We previously reported the first turkey reovirus S3 sequence for NC 98 (6). The turkey reoviruses present here have a 96% amino acid identity to NC 98, based on amino acid sequence. Phylogenetic analysis of the σ2 amino acid sequences demonstrated that the turkey reoviruses form a distinct, separate clade from their avian counterparts within subgroup 2 of the *Orthoreovirus* genus. This was supported by high bootstrap confidence levels, and the same relationships were identified when comparing nucleotide sequences. Based on sequence analysis of the σ2 gene, turkey reovirus isolates appear to be a different virus species according to the guidelines set forth by ICTV (8) from chicken, duck, and NBV isolates within the subgroup 2 clade.

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